

Forum Review

Pharmacologic Induction of Heme Oxygenase-1

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ABSTRACT

Heme oxygenase-1 (HO-1) is a cytoprotective protein whose expression is consistently associated with therapeutic benefits in a number of pathologic conditions such as atherosclerotic vascular disease and inflammation. Although the expression of HO-1 in most tissues is low, a large number of clinical and experimental pharmacologic compounds have been demonstrated to induce HO-1. This induction is suggested to be at least partially responsible for the perceived therapeutic efficacy of these compounds. The increase in HO-1 expression in response to these compounds is the result of a complex regulatory network involving many signaling pathways and transcription factors. Understanding both the pathways by which HO-1 is induced and the mechanism through which the enzyme exerts its beneficial effects may facilitate the development of novel drugs. *Antioxid. Redox Signal.* 9, 2227–2239.

INTRODUCTION

SOME 30 YEARS AGO, Tenhunen *et al.* (121) were the first to describe heme oxygenase activity (121). Since then, an explosive growth has occurred in our knowledge in this field (81). Heme oxygenase catalyzes the first and rate-limiting step of heme metabolism (139), by binding heme as a prosthetic group and as a substrate. The reaction involves seven steps, consumes molecular oxygen and NADPH and requires cytochrome P-450 reductase activity (77), and generates stoichiometric amounts of carbon monoxide, Fe²⁺, and biliverdin as the products (Fig. 1). The mechanism of HO-1-catalyzed degradation of heme has been comprehensively reviewed (94). In mammals, biliverdin is then transferred to the ubiquitously expressed biliverdin reductase, which reduces the pigment to bilirubin (129). This is an unusual step, as it represents the only anabolic reaction known in intermediary metabolism.

Two functional heme oxygenase isoforms are expressed in mammals (103). In most tissues, HO-1 is either expressed at low levels or not expressed at all, whereas heme oxygenase-2 is constitutively expressed. Although the expression of HO-

1 can be induced in response to a range of stimuli, heme oxygenase-2 responds to few regulatory factors (80). The differential expression profile suggests that these two isoforms carry out different functions *in vivo*. Interestingly, the research on HO-1 has undergone a gradual shift of focus, from its role as a crucial enzyme with a unique metabolic function to its modulatory effect on cell survival and proliferation (30). It is now well established that HO-1 expression protects cells from physical, chemical, and biologic stress by bringing about subtle changes in cellular physiology (104). It has been suggested that the induction of HO-1 by pharmacologic compounds contributes to at least some of the perceived efficacy of these therapeutics (Fig. 2) (4). Specifically, the product(s) of the activity of HO-1 are now commonly regarded as the protective effector molecules. This is a remarkable shift, given that carbon monoxide and bilirubin have long been considered to be toxic waste products (114). This review aims to provide a summary of the current understanding of HO-1 as a target of clinical and experimental therapeutic compounds, as well as to comment on yet unresolved and controversial issues in this field.

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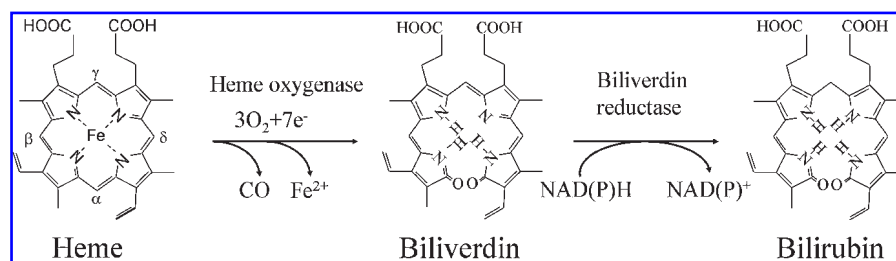


FIG. 1. Reaction scheme of oxidative metabolism of heme. Oxidative heme metabolism catalyzed by heme oxygenase sequentially yields CO, ferrous iron, and biliverdin. The electrons required for this reaction are supplied by cytochrome P-450 reductase. In mammals, biliverdin is reduced subsequently to bilirubin by biliverdin reductase and at the expense of NADPH.

MOLECULAR REGULATION OF HO-1 EXPRESSION

The control of HO-1 expression occurs primarily at the transcription level (17). Analysis of the HO-1 gene structure revealed a number of transcription factor binding sites in the 5'-flanking region of the promoter. Transcription factors such as nuclear factor E2-related factor-2 (Nrf2), activator protein-1 (AP-1), and nuclear factor-kappa B (NF- κ B) bind to specific sequences within two enhancer elements (E1 and E2), resulting in the upregulation of HO-1 expression (58). Within both E1 and E2 regions of the HO-1 gene, multiple stress-response elements are found. This explains the potent induction of HO-1 by most agents that cause cellular stress (100). Selected therapeutic compounds also induce HO-1 by activating transcription factors, often *via* upstream signaling pathways. For some pharmacologic compounds, the transcription factor(s) responsible for HO-1 induction is(are) not yet identified. Nevertheless, it is increasingly clear that HO-1 induction by therapeutic compounds often requires a complex set of molecular events in a tissue-specific manner, sometimes involving two or more signaling pathways or transcription factors working synergistically (95). A brief discussion follows of several key transcription factors involved in HO-1 regulation.

Nrf2

Nrf2 is a noted cellular regulator of antioxidant and stress response because of its affinity for the antioxidant response elements (1). Antioxidant response elements are found in the regulatory region of a number of genes involved in detoxification of xenobiotics (phase II response genes) and antioxidant defense (90). Apart from HO-1, other genes believed to be controlled by Nrf2 include NAD(P)H:quinone oxidoreductase, glutathione *S*-transferase, and thioredoxin (60). How Nrf2 is activated by external stimuli is incompletely understood and remains an active area of research. The regulation of Nrf2 activation occurs at multiple levels. Under basal conditions, Nrf2 is sequestered in the cytoplasm, tethered to binding partner Kelch-like ECH-associated protein-1 (Keap1) (61). By actively promoting its degradation, presumably *via* ubiquitination of Nrf2 by Keap1-associated ubiquitin ligase, such as Cul3, Keap1 prevents the translocation of Nrf2 into the nucleus (112). Keap1 is a cysteine-rich protein whose thiol groups are prone to oxidative modification (127). Reaction of these thiol groups with oxidants or electrophiles is thought to lead to confor-

mational changes in Keap1 protein, such that Nrf2 dissociates from the complex and translocates to the nucleus (Fig. 3) (32).

In the nucleus, Nrf2 competes with the inhibitory Bach-1 protein for binding to the small Maf protein heterodimer (27). Heme interacts with Bach-1 and renders it incapable of binding Maf proteins (115). Another strong HO-1 inducer, cadmium, promotes the nuclear export of Bach-1 (117). Under these conditions, Nrf2/small Maf protein heterodimer binds to the ARE and enhances gene expression. It is unclear whether other known activators of Nrf2 cause Bach-1 displacement.

AP-1

AP-1 is a family of transcription factors consisting of homo- or heterodimers of basic leucine zipper proteins from the Fos, Jun, activating transcription factors, and Maf protein families (67). Similar to Nrf2, AP-1 activation can also be modulated by cellular oxidative status. For example, antioxidant treatment of cells increases the expression of c-Fos and c-Jun (20).

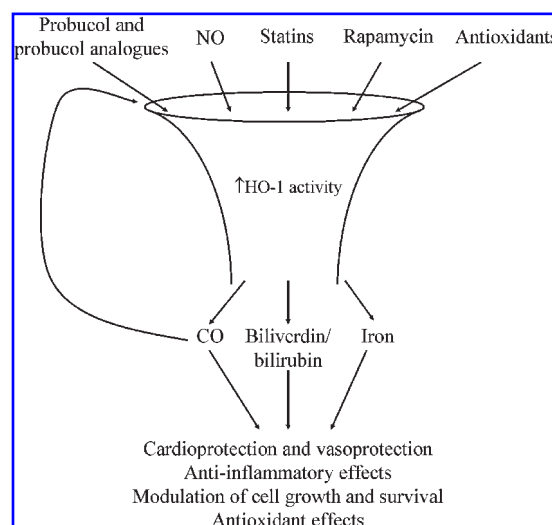


FIG. 2. The concept of HO-1 as a therapeutic funnel. In this hypothesis, various classes of therapeutic compounds induce HO-1 expression and activity. The products of this enzymatic reaction then mediate the antiinflammatory, antioxidant, and/or growth-modulating activities associated with increased HO-1 expression. Some of the products, such as CO, themselves increase HO-1 expression, thereby amplifying the biologic effect. Adapted from ref. 4.

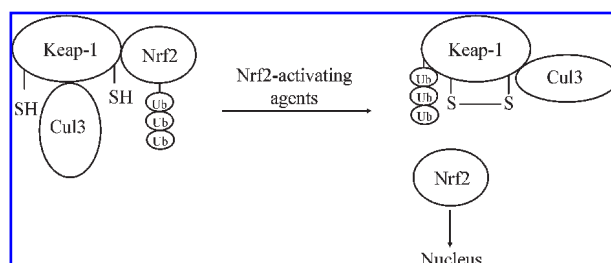


FIG. 3. Keap1/Nrf2 interaction. Under normal condition, Keap1 sequesters Nrf2 in the cytosol of cells, and ubiquitin ligases associated with Keap1 promote ubiquitination of Nrf2 and its subsequent degradation. A large number of chemicals are thought to modify the thiol group of specific cysteine residues of Keap1 (shown here schematically as oxidation with formation of a disulfide bond), leading to conformational change in the protein and the ubiquitination of Keap1 instead of Nrf2. As a result, Nrf2 dissociates from the complex and can translocate to the nucleus. Ub, ubiquitin; Cul, Keap1-associated ubiquitin ligase.

AP-1 mediates the upregulation of HO-1 by a range of stimuli including heme, arsenite, and heavy metals. Because Maf proteins can also form part of the AP-1 complex, dissociation of Bach-1 from these proteins would enhance the activity of AP-1 as well as that of Nrf2.

NF- κ B

NF- κ B is a family of transcription factors with important functions in diverse physiologic and pathologic processes such as inflammation, apoptosis, and cancer progression (66). Similar to the situation with Nrf2, NF- κ B is sequestered in the cytosol under normal conditions by a member protein of the inhibitor of kappa B family (6). On stimulation by external factors, the inhibitor of kappa B becomes phosphorylated by several kinases, such as the inhibitor of kappa B kinase and mitogen-activated protein kinase (65). Phosphorylated inhibitor of kappa B is then degraded by the proteasome in a ubiquitin-de-

pendent process (97), allowing NF- κ B to migrate to the nucleus. NF- κ B binding to enhancer elements increases HO-1 expression as a rapid response to cellular injury (74).

Apart from the three transcription factors discussed earlier, several others enhance HO-1 expression. These include cAMP-responsive element-binding proteins (70) and the Ets family of transcription factors (22). The large number of transcription factors that can upregulate HO-1 expression likely contribute to the highly inducible nature of the gene.

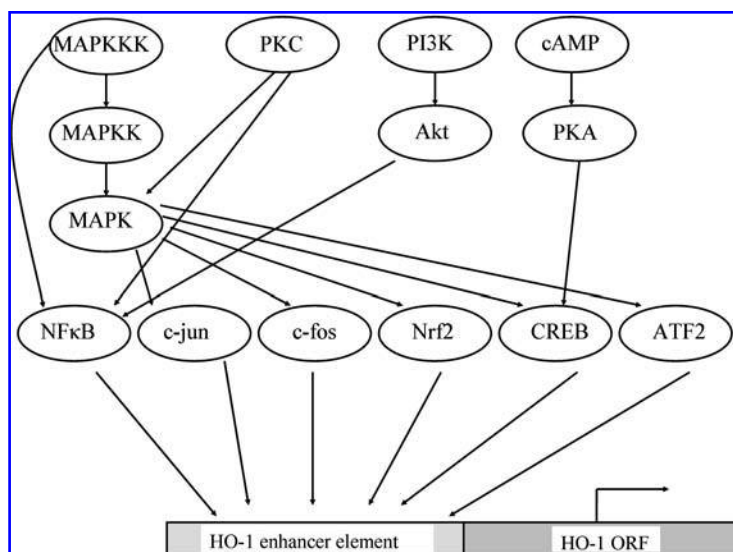
SIGNALING PATHWAYS

Activation of transcription factors is often the last step in complex intracellular signaling cascades leading to an increase in HO-1 mRNA synthesis. Several important pathways involving serine/threonine kinases mediate the induction of HO-1 in response to external stimuli (99) (Fig. 4).

Mitogen-activated protein kinase (MAPK) families, including p38 MAPK, extracellular signal-regulated protein kinase, and c-Jun N-terminal kinase, are serine/threonine kinases activated by external stimuli (13). A cascade of three sets of kinases controls the activity of MAPK (62). The exquisite regulation of MAPKs enables them to produce a precise, cell-specific effect in response to a wide array of stimuli. In general, extracellular signal-regulated protein kinases are activated by external growth signals, whereas p38 and c-Jun N-terminal kinase respond to external stress.

Although it is clear that MAPKs play a major role in mediating HO-1 upregulation, the exact underlying mechanism is less certain. Various MAPKs have been shown directly to phosphorylate or indirectly to regulate transcription factors important for upregulating HO-1. Thus, AP-1, Nrf2, NF- κ B, and cAMP-responsive element-binding proteins are all targets of the MAPKs (124). However, whether these interactions are sufficient to mediate HO-1 expression is unknown. Notably, the role of p38 MAPK in regulating HO-1 expression is unclear. In apparent contrast to previous works (2), a recent study found p38-dependent phosphorylation of Nrf2 to enhance its interac-

FIG. 4. Some signaling pathways leading to HO-1 induction. Activation of a number of transcription factors as a result of cellular signaling events brings about the increase in HO-1 expression. Serine/threonine kinases are especially prominent in responding to external and internal stimuli leading to HO-1 induction. MAPKKK, MAP kinase kinase kinase; MAPKK, MAP kinase kinase; ATF-2, activating transcription factor 2.



tion with Keap1 and thereby to decrease its translocation to the nucleus (68).

Protein kinases such as protein kinases A and G are regulated by changes in cellular concentration of cAMP and cyclic guanosine monophosphate (cGMP), respectively. These cyclic nucleotides bind to their respective protein kinase, thereby inducing a conformational change and kinase activity (35). It has been shown that signaling *via* protein kinases A and G can lead to the expression of HO-1 (57). Other protein kinases, such as protein kinase C and phosphatidylinositol 3-kinase/Akt have also been implicated in signaling pathways leading to HO-1 induction (122).

The molecular and cellular pathways described earlier should not be considered separately. Instead, they should be viewed as an array of interconnecting signals, all contributing to the final outcome, thereby allowing fine control of the duration and extent of HO-1 induction.

INDUCTION OF HEME OXYGENASE-1 BY PHARMACEUTICAL COMPOUNDS

As mentioned earlier, the expression of HO-1 can be induced by an ever-increasing list of compounds, some of which have therapeutic properties. Research over the past decade has focused on several classes of drugs (Table 1).

Drugs acting on the cardiovascular system

Cardiovascular diseases are leading causes of mortality and morbidity in the developed world (79). Compelling evidence from knockout animal models, pharmaceutical interventions, and gene-transfer experiments has established clearly that the expression of HO-1 in vascular cells confers protection against atherosclerotic vascular disease (for a recent review, see ref. 113). HO-1 attenuates the development of atherosclerotic lesions (59, 64), reduces restenosis and vascular constriction (29, 125), ameliorates myocardial ischemia injuries (138), and promotes vessel relaxation (105). Not surprisingly, induction of HO-1 as a way to control and treat atherosclerotic vascular diseases is an area of intense research. Several compounds with established antiatherogenic or cardioprotective activities are able to induce HO-1.

Statins. Statins are a class of competitive inhibitors of 3-hydroxy-3-methylglutaryl co-enzyme A reductase, a rate-limiting enzyme of cholesterol biosynthesis (33). Overwhelming evidence from many independent trials indicates that statins are effective in reducing the incidence of cardiovascular events and associated mortality. Grosser and colleagues (42) demonstrated that several statins at mid to high micromolar concentrations induce HO-1 expression in ECV304 (42), a human epithelial cell line (12). Two *in vivo* studies subsequently confirmed simvastatin to induce HO-1 mRNA and activity in vascular smooth muscle cells and heart (52, 75). This induction was tissue specific (with smooth muscle cells of the vasculature as the major target) and associated with potentially antiatherogenic activities, such as attenuation of smooth muscle cell proliferation and inhibition of NF- κ B activation (75). A similar HO-1-inducing

effect was observed in macrophages treated with statins (15). Further studies in human cases of atherosclerotic vascular diseases with clinically relevant doses will be required to address whether statins exert some of their vasoprotective effect *via* HO-1 induction. Specifically, the concentrations of statins used in the aforementioned experimental studies were much higher than peak serum concentration achieved and doses commonly used in clinical practice (9). In this context, it is noteworthy that atorvastatin at pharmacologically relevant concentrations has been recently reported to restore hypoxia-driven inhibition of endothelial nitric oxide synthase without affecting HO-1 in human endothelial cells (78).

Nitric oxide and nitric oxide-releasing compounds. Nitric oxide (NO) is an important signaling molecule with a diverse range of functions in many cell types (54). Initially regarded as a toxic gas, NO is now recognized as a powerful mediator of vascular function because of its ability to activate soluble guanylyl cyclase (sGC) and thereby increase the synthesis of the second-messenger molecule cGMP (56). NO is a prominent regulator of physiologic and pathologic activities contributing to vascular homeostasis and function. Endothelial cell-derived NO is a major vasodilator, primarily responsible for determining basal vascular tone (53). Additionally, vascular NO production affects processes such as platelet aggregation (85), leukocyte trafficking (48), and the proliferation and migration of smooth muscle and endothelial cells (39). Decreased NO bioavailability is an early symptom of atherosclerotic vascular disease (88), and organic nitrates and nitrites that release NO are used clinically to relieve symptoms of myocardial ischemia, most commonly, angina pectoris. The therapeutic effect of these compounds is achieved by relaxation of affected vessels mediated *via* NO, sGC, and cGMP (55).

The group of Motterlini (86) was the first to discover that NO-releasing compounds, such as sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine, and 3-morpholinosydnonimine, induce HO-1 in endothelial cells (86). Subsequently, induction of HO-1 was demonstrated in different cell types with other NO-releasing compounds (31, 47, 137) and pure NO gas (11). Activation of sGC and a subsequent increase in cGMP is believed to be a major pathway leading to HO-1 induction by NO and NO-releasing compounds, although an increase in HO-1 mRNA stability may also contribute (82).

In addition to organic nitrates, other therapeutic compounds may induce HO-1 *via* the NO-sGC-cGMP pathway by increasing NO production *via* activation of nitric oxide synthase (NOS). Aspirin is an antithrombotic drug whose pharmacologic effect stems from its inhibition of cyclooxygenase. Aspirin acts on both isoforms of cyclooxygenase, cyclooxygenase-1 and -2, and interferes with enzyme activity by acetylating a serine residue near the active site (73). This covalent modification prevents the conversion of arachidonic acid to prostaglandins that are powerful mediators of inflammation and thrombosis (84). Aspirin was reported to induce HO-1 in human epithelial ECV304 cells (41). Whether this contributes to the protective action of aspirin remains to be established, because drug concentrations of ≥ 1 mM were required to increase HO-1 activity. Notwithstanding this, HO-1 induction by aspirin appeared to be dependent on NO synthesis, as an NOS inhibitor blocked the process (41). Consistent with this, 15-

epi-lipoxin-A4, which is produced by cyclooxygenase from arachidonic acid in the presence of aspirin (23), increases NO synthesis by activating endothelial and inducible NOS (96), and nanomolar concentrations of a stable analogue of 15-epi-lipoxin-A4 induce HO-1 (89). These studies suggest that aspirin may exert part of its antiinflammatory effect *via* NO-mediated induction of HO-1.

Another cyclooxygenase inhibitor currently undergoing pre-clinical trials, AZD3582, also induced HO-1 expression in two epithelial cell lines (10). AZD3582 is metabolized to NO and naproxen. As naproxen does not induce HO-1, it was speculated that AZD3582 induces HO-1 *via* the NO-dependent pathway (10).

Probucol and probucol analogues. Probucol, a rarely used cholesterol-reducing drug, has been reported to inhibit human atherosclerosis (107) and restenosis after coronary angioplasty with (120) and without stenting (119). Probucol also retards experimental atherosclerosis (132) and intimal thickening after balloon injury (29). Considerable evidence now indicates that upregulation of HO-1 plays a key role in these beneficial activities of probucol against atherosclerotic vascular disease (133). Thus, *in vivo* treatment with probucol induces HO-1 in vascular smooth muscle cells. This is associated with attenuation of smooth muscle cell proliferation and intimal hyperplasia, and a promotion of reendothelialization and restoration of endothelium-dependent relaxation of the injured blood vessel. All of these beneficial activities of probucol are blocked when animals are co-treated with tin-protoporphyrin IX, a pharmacologic inhibitor of heme oxygenase. *In vitro* studies have established that probucol induces HO-1 mRNA, protein, and activity in vascular smooth muscle cells, and this results in inhibition of cell proliferation (26). Inhibition of heme oxygenase by tin-protoporphyrin IX, or specific blockade of HO-1 with siRNA, completely prevented the ability of probucol to inhibit smooth muscle cell proliferation (26).

As indicated earlier, in humans, probucol effectively inhibits restenosis after angioplasty (119). Surprisingly, this benefit was nullified in subjects receiving probucol together with an “antioxidant” cocktail, consisting of vitamins C and E and β -carotene (119). Consistent with the hypothesis that HO-1 induction, rather than antioxidant activity, is the primary determinant of success in pharmacologic intervention by probucol, vitamin E failed to induce HO-1 in vascular smooth muscle cells (133). Furthermore, ascorbic acid has been reported to attenuate upregulation of HO-1 in macrophages induced by oxidized low-density lipoprotein (45). We recently tested the effect of the antioxidant cocktail on the ability of probucol to induce HO-1 mRNA in smooth muscle cells *in vitro*. We observed that the inclusion of the antioxidant cocktail did not change the extent to which probucol induced HO-1 (Fig. 5). This may relate to potential differences in results obtained *in vitro* versus *in vivo* (see later). In this context, it will be interesting to investigate whether the probucol analogue, AGI-1067, currently in phase III clinical trial as an antiatherosclerosis drug, induces HO-1 and, if so, whether this contributes to its beneficial activities. Based on *in vivo* structure–function studies performed in rabbits, one would predict AGI-1067 to induce HO-1 activity, as AGI-1067 contains the sulfur groups required for HO-1 induction *in vivo* (133). Conversely, one would predict

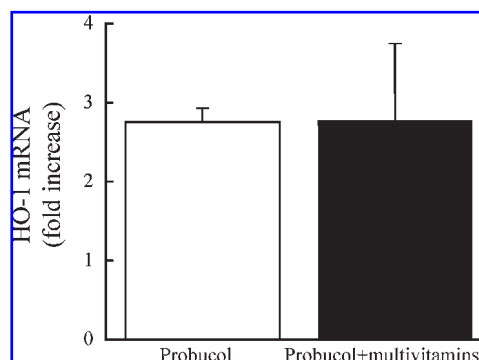


FIG. 5. Multivitamins do not independently induce HO-1. Rat aortic smooth muscle cells were treated with probucol (100 μ M) or a combination of probucol and multivitamins (100 μ M ascorbate, 60 μ M α -tocopherol, and 1 μ M β -carotene) for 2 h. The relative mRNA level of HO-1 was determined by quantitative real-time PCR, as described (26). Results show mean \pm SEM ($n = 3$).

that the probucol ether analogue, BM 15.0639, does not induce HO-1 *in vivo*, as it lacks the active sulfur atoms and fails to inhibit experimental atherosclerosis (36).

Vascular-active peptides. A few naturally occurring peptides with vascular activity were reported to induce HO-1. Adrenomedullin, a vasodilatory peptide secreted by vascular cells, induce HO-1 by an NO-dependent mechanism (102). Adrenomedullin also modulates cellular cAMP content, suggesting that the peptide may induce HO-1 by two distinct mechanisms (102). Atrial natriuretic peptide, another cardiovascular hormone with antiinflammatory activities, also induces HO-1 in cultured proximal renal tubular epithelial cells *via* an NO-dependent pathway (98). Interestingly, in endothelial cells, atrial natriuretic peptide appears to induce HO-1 *via* extracellular signal-regulated protein kinase and c-Jun N-terminal kinase, suggesting cell-type–specific activation pathways (69).

D-4F is currently being developed as an antiatherogenic drug. D-4F is a synthetic mimetic peptide of apolipoprotein A-I, the major protein component of high-density lipoprotein. In a rat model of diabetes, D-4F potently induced HO-1 in the aorta, leading to increased bilirubin production (71). D-4F also decreased the vascular level of superoxide anion radical and endothelial cell sloughing, and it improved vessel relaxation (71). However, a causal link between HO-1 induction and the beneficial effect of D-4F remains to be established.

Polyphenols and Keap1-modifying agents

Although HO-1 is induced potently in response to oxidative stress, certain polyphenols with antioxidant activity or thiol-modifying agents can also increase its expression (93). This class of compounds includes many different chemicals (Fig. 6), and this chemical difference is reflected in the diverse upstream pathways implicated in HO-1 induction. Many antioxidants capable of inducing HO-1 contain phenol or reactive sulfur groups, and they potently activate Nrf2 by disrupting its inter-

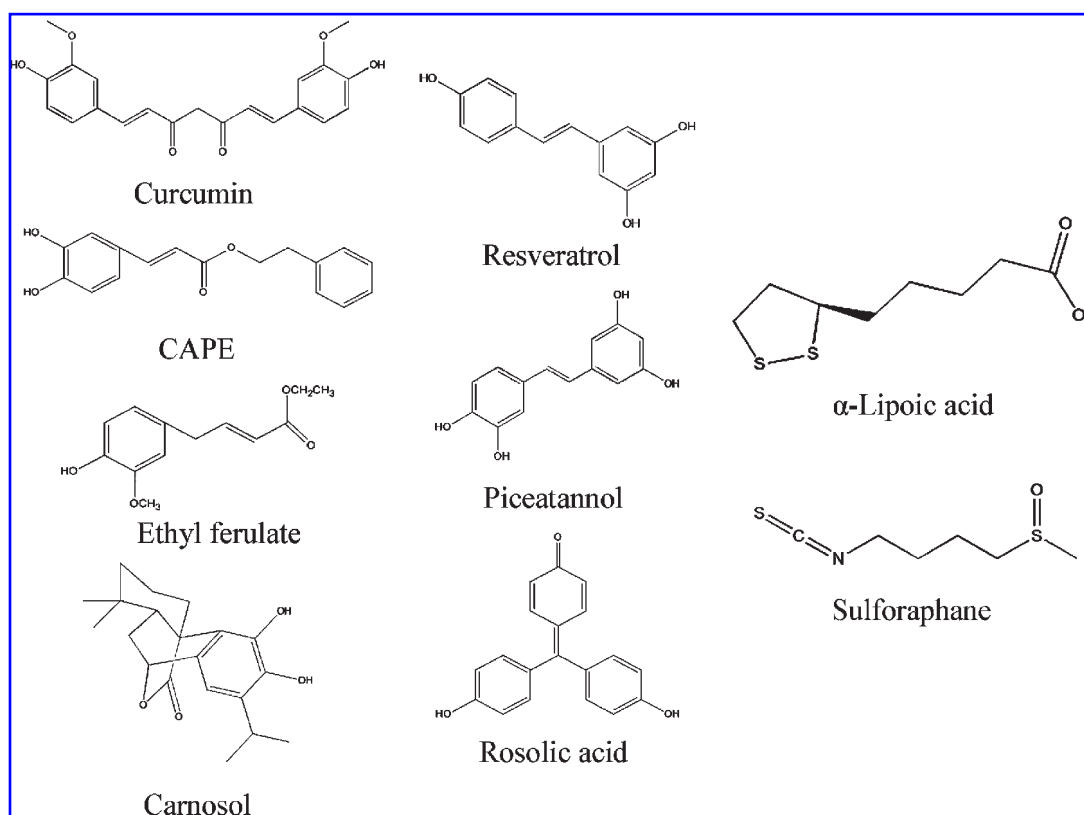


FIG. 6. Chemical structures of selected HO-1 inducers. Inducers of HO-1 include a broad range of different chemicals. CAPE, caffeic acid phenethyl ester.

action with Keap1 (51). The antineoplastic action of these compounds is largely attributable to Nrf2-mediated upregulation of phase II detoxification enzymes.

Keap1-modifying compounds. Some polyphenols can act as Michael reaction acceptors (28) and modify specific cysteine residues in Keap1 (127), thereby leading to the nuclear localization of Nrf2 and increased transcription of HO-1. Curcumin, caffeic acid phenethyl ester, and rosolic acid are examples of this class of compounds (see Fig. 6).

Curcumin is currently being investigated for anticancer and antiinflammatory activities (72). This diphenol contains unsaturated α,β -unsaturated carbonyl moieties, which react with nucleophiles such as cysteine thiol groups. Curcumin was first shown to increase HO-1 expression in bovine aortic endothelial cells, and Nrf2 was identified as the transcription factor responsible for this induction (87). Subsequently, several reports confirmed that curcumin caused Nrf2-Keap1 dissociation and binding of Nrf2 to the ARE in a number of cell types (49). Concentrations of curcumin that induced HO-1 (*i.e.*, 15 or 30 μ M) also caused a transient increase in cellular glutathione (108). In contrast, higher concentrations of curcumin (*i.e.*, ≥ 50 μ M) were unable to induce HO-1 and were associated with cell death. *N*-Acetyl cysteine did not abolish HO-1 induction by curcumin, although it prevented cell death resulting from high-dose curcumin. The authors interpreted these results as suggestive of selected, rather than general cysteine modification(s) being required for Nrf2 activation (108). Both p38 MAPK and NF- κ B, but not extracellular signal-regulated protein kinase, c-Jun N-

terminal kinase, and the phosphatidylinositol 3-kinase pathway, were shown to be involved in increased Nrf2 activity (3). Rosolic acid, a plant triphenylmethane, has also been shown to induce HO-1 in endothelial cells (34).

Caffeic acid phenethyl ester, a phenol isolated from the honeybee *propolis*, has anticancer properties and is structurally similar to curcumin. Caffeic acid phenethyl ester induces HO-1 in astrocytes in much the same way as curcumin (108), although the former is comparatively more toxic. Similar to curcumin, caffeic acid phenethyl ester can potentially inhibit NF- κ B. To reconcile this feature with the observation that NF- κ B activation is required for curcumin- and (by inference) caffeic acid phenethyl ester-mediated HO-1 induction, it was suggested that low doses of these compounds do not inhibit NF- κ B, and that this also explains why high concentrations of the compounds do not induce HO-1 (108).

Sulforaphane is a naturally available sulfur-containing compound found in crucifers such as broccoli. It is noted for its chemopreventive effects and is known to induce a phase II response while inhibiting a phase I response (101). Sulforaphane is a strong activator of Nrf2. Recent studies have identified the isothiocyanate group of sulforaphane to modify cysteine residues of Keap1 (50). Interestingly, this modification is distinct from previously studied ARE inducers that modify Keap1 by alkylation (50). Sulforaphane treatment of cells does not lead to accumulation of ubiquitinated Keap1 (140), suggestive of an alternative activation pathway of HO-1. Sulforaphane was shown to increase HO-1 protein in aortic smooth muscle cells isolated from spontaneously hypertensive rats (134). Concur-

rent with this induction, the activity of other Nrf2-regulated genes, such as glutathione peroxidase and glutathione-S-transferase, was also increased (134).

Activators of Nrf2. Some compounds activate Nrf2 *via* various signaling pathways rather than *via* direct reaction with Keap1. For example, carnosol, a phenolic antioxidant found in rosemary, was reported to induce HO-1 in PC12 neuronal cells *via* Nrf2 activation (83). The phosphatidylinositol 3-kinase/Akt pathway was implicated in this induction, possibly by signaling to several downstream ubiquitin ligases that modify Keap1. Similarly, the sulfur-containing phytochemical α -lipoic acid induces HO-1 in the human monocytic cell line THP-1, albeit at rather high concentrations (≤ 1 mM) (92). This upregulation was largely abolished in cells overexpressing a dominant-negative Nrf2 gene, or cells treated with a p38 MAPK inhibitor. Inhibition of p38 prevented Nrf2 binding to the ARE, although the underlying molecular mechanism remains unclear.

Other polyphenols. Resveratrol is present in grapes and red wine (24) and is now available as a nutrient supplement. Its anticancer properties are well documented (7). Resveratrol is thought to inhibit cancer development by acting on multiple targets, including inhibition of angiogenesis (16) and carcinogen biotransformation by phase I enzymes (21). Resveratrol induces apoptosis by modulating important regulatory molecules, such as p53, Fas/FasL, and cyclins (25). The cardioprotective properties of resveratrol have also been investigated *in vitro* and *in vivo*. Resveratrol has been reported to ameliorate myocardial ischemia–reperfusion injury by multiple mechanisms (40) and to enhance angiogenesis after myocardial infarction (38). Its impact on atherosclerosis is not clear, with one study reporting an adverse effect in hypercholesterolemic rabbits (131), and another study observing a beneficial activity in hypercholesterolemic mice (37). Resveratrol has been repeatedly shown to prolong the life span of several organisms, including mice (8).

The ability of resveratrol to induce HO-1 was discovered only recently in vascular smooth muscle cells and PC12 neurons (14). This induction appears to be cell-type and concentration specific, as other studies found no evidence for HO-1 induction by resveratrol in some other cell types (108, 136), or observed resveratrol to increase HO-1 mRNA, without corresponding increases in protein or heme oxygenase activity (109). How resveratrol induces HO-1, and whether it can directly modify Keap1, also remains unclear.

Piceatannol, an antiinflammatory agent structurally related to resveratrol, was recently shown to induce HO-1 in endothelial cells (136). The key signaling molecules responsible for this upregulation were found to be PKC and tyrosine kinase.

Antineoplastic agents. Paclitaxel is used clinically with cisplatin for combined therapy against several types of cancer because of its ability to interfere with disassembly of microtubules. Paclitaxel has been reported to induce HO-1 in smooth muscle cells, which may explain the drug's inhibitory effect on proliferation in these cells (18). Paclitaxel is being trialed on drug-eluting stents to prevent restenosis (118). Overexpression of HO-1 has been associated with increased survival of cancerous cells, mainly because of prevention of apoptosis (130). It is therefore difficult to see how induction of HO-1 con-

tributes to the efficacy of these antineoplastic compounds. Several phytochemicals discussed earlier also possesses antiinflammatory or antioxidant activities or both, and could conceivably be adapted to treat diseases other than cancer.

Drugs acting on the immune systems

Rapamycin, used under the trade names Sirolimus and Rapamune, is a macrocyclic lactone initially isolated from *Streptomyces hygroscopicus* and used clinically as an immunosuppressive drug. Rapamycin binds to immunophilin FKBP-12, and the complex then binds to and inhibits mammalian kinase, target of rapamycin (135). Rapamycin demonstrates antiproliferative properties against vascular endothelial and smooth muscle cells, and it effectively reduces the incidence of restenosis (128). Rapamycin induces HO-1 in pulmonary endothelial and smooth muscle cells *in vitro* (126), and good evidence suggests that this contributes to the antiproliferative activities of rapamycin. Thus, rapamycin-mediated inhibition of proliferation is abrogated in smooth muscle cells isolated from HO-1^{-/-} mice, or when cells from wild-type mice are treated with tin-protoporphyrin IX (141). The signaling pathway(s) involved in this HO-1 induction remain unclear. In contrast to rapamycin, cyclosporin A, another immunomodulatory drug, does not induce HO-1 (126).

Other compounds

Other phenols capable of inducing HO-1 include the phenol ethyl ferulate (109), and 1,2,3,4,6-penta-*o*-galloyl-beta-D-glucose (19). It is speculated that Nrf2 is involved in induction of HO-1 by these compounds.

Pharmacologic doses of insulin have been reported to induce HO-1 in renal cells *via* the phosphatidylinositol 3-kinase/Akt pathway and Nrf2 (46), and this may represent a mechanism by which insulin protects the kidney in addition to its effect on circulating glucose concentration.

A clinical β -adrenergic receptor agonist, isoproterenol, induces HO-1 in a murine macrophage cell line (116). This process re-

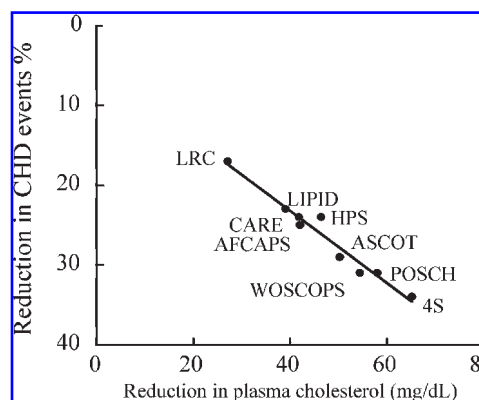


FIG. 7. Linear correlation between serum cholesterol reduction and reduction in coronary heart disease (CHD) events. Results from nine large-scale clinical studies suggest that cholesterol reduction is the single most important factor contributing to clinical benefits of statins. Adapted from refs. 43, 110, and 123.

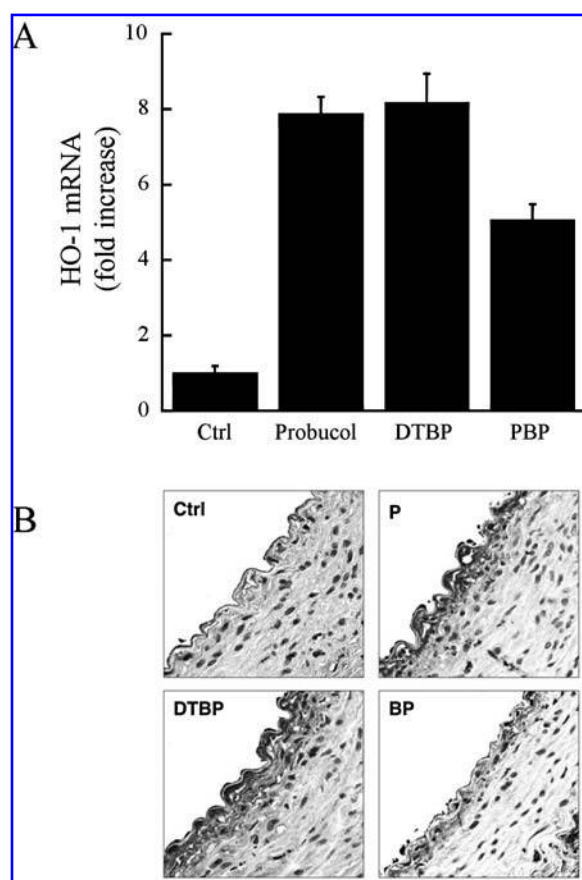


FIG. 8. Probulcol bisphenol induces HO-1 *in vitro* but not *in vivo*. (A) Rabbit aortic smooth muscles cells were treated with 100 μ M either probucol, probucol dithiobisphenol (DTBP), or probucol bisphenol (PBP) for 24 h before the relative level of HO-1 mRNA was determined by quantitative real-time PCR, as described (26). Results show mean \pm SEM ($n = 3$). $*p < 0.05$ compared with control (Ctrl); Mann-Whitney Rank Sum test. (B) Immunohistochemical detection of HO-1 protein 4 days after balloon injury in aortic smooth muscle cells of rabbits supplemented with probucol, probucol dithiobisphenol, or probucol bisphenol. Comparable results were obtained for aortic tissue HO-1 mRNA and heme oxygenase activity (not shown). Reproduced with permission from ref. 133.

quires β_1 -adrenergic receptor and PKA signaling. However, it is not clear how these pathways bring about HO-1 induction, nor is obvious physiologic relevance associated with this process.

POTENTIAL PITFALLS

One potential pitfall in the process of identifying HO-1 inducers as therapeutic compounds lies in the fact that HO-1 can be induced both as an endogenous response to stress and as a therapeutic defense against various pathologic conditions (76). At sufficiently high dose, most drugs are harmful or toxic, even though obvious signs of cell stress such as cell death may not be apparent. In some cases, toxicity occurs even at very low doses, such as submicromolar concentration in the case of rapamycin (111). Potentially beneficial induction of HO-1 must

therefore be distinguished from HO-1 upregulation as a response to stress brought about by toxic concentrations of a particular drug. This requires careful selection of experimental conditions. It also requires relevant consideration of the known pharmacokinetic properties of the compounds. In some cases, external stress may induce HO-1 indirectly by increasing available heme as a result of increasing cell death or cell turnover.

Caution must also be exercised when linking the HO-1-inducing properties of therapeutic compounds to their clinical efficacy. For example, metal protoporphyrins that are widely used as heme oxygenase inhibitors are well known to be nonspecific, particularly when used at high concentrations (44). In most cells, the heme available for heme oxygenase is very low and well below the K_m of HO-1 (106), so that an increase in HO-1 content may not necessarily lead to increased production of CO and biliverdin/bilirubin. In addition, induction of HO-1 may not be the primary mechanism by which a therapeutic agent provides clinical benefit. For example, and as mentioned earlier, statins induce HO-1 in various cell types, and this has been proposed to account for their "pleiotropic" effects. However, with regard to coronary heart disease, little evidence exists that these pleiotropic effects are clinically relevant. Indeed, results of a number of large-scale studies have established that the reduction in incidence of coronary events after treatment with statins is almost entirely attributable to the extent of reduction in cholesterol, and for a given extent of cholesterol reduction, the efficacy of statins is comparable to that of other cholesterol-reducing therapies (Fig. 7). This argues against a clinically significant role of HO-1 induction by statins, as in this case, one would have expected statins to reduce coronary heart disease events to a greater extent than other cholesterol-reducing therapies.

Many therapeutic compounds are delivered as prodrugs that undergo modification *in vivo* to the active form. Simvastatin

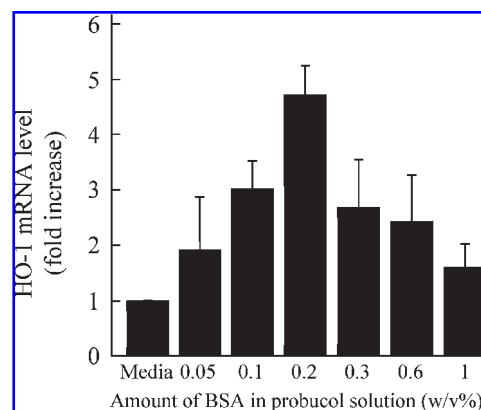


FIG. 9. The amount of carrier protein-to-drug ratio affects the ability of probucol to induce HO-1 *in vitro*. Probulcol (final concentration, 100 μ M) was presented to rat aortic smooth muscle cells as a complex with bovine serum albumin at the amount indicated. The relative level of HO-1 mRNA was determined by quantitative real-time PCR 2 h after commencement of treatment, as described previously (26). The extent of HO-1 induction appeared maximal at 0.2% (wt/vol) albumin, corresponding to a molar albumin-to-probulcol ratio of $\sim 1:3$. Results show mean \pm SEM ($n = 3$ separate experiments each done in triplicate, except for 0.2% BSA, which is $n = 1$), with the extent of HO-1 expression expressed relative to the no probucol-containing sample (*i.e.*, medium).

TABLE 1. SOME PHARMACOLOGIC INDUCERS OF HO-1

Inducer	Cells	Nrf2 Activation	References
Statins*	ECV304 Human epithelial cell line	Uncertain	42
	RAW264.7 Mouse macrophage cell line	Uncertain	15
	Human and rat aortic smooth muscle cells	Uncertain	75
NO donors	Porcine aortic endothelial cells	No	86
	Rat aortic smooth muscle cells		31,47
Organic nitrates	ECV304 Human epithelial cell line	No	91
NO	Human embryonic lung fibroblasts	No	82
Aspirin	ECV304 Human epithelial cell line	No	41
15-Epi-lipoxin-A ₄ analogue	ECV304 Human epithelial cell line	No	89
AZD3582	ECV304 Human epithelial cell line, KATO III human gastric adenocarcinoma cells	No	10
Probucol	Rabbit aortic smooth muscle	No	26
Adrenomedullin	Rat smooth muscle cells	No	102
Atrial natriuretic peptide	LLC-PK1 Pig proximal renal tubular epithelial	No	98
	Human umbilical vein endothelial cells	No	69
D-4F	Rat aorta	No	71
Curcumin	Human and porcine renal proximal tubule cells	Yes	5
	Bovine aortic endothelial cells		87
	HBL100 and MDA-MB468 human breast cells		49
	DI TNC1 Rat astrocytes		108
Rosolic acid	Bovine aortic endothelial cells	Yes	34
CAPE	DI TNC1 Rat astrocytes	Yes	108
Sulforaphane	Rat aortic smooth muscle cells	Yes	134
Carnosol	PC12 rat pheochromocytoma	Yes	83
α -Lipoic acid	THP1 human monocytic cells	Yes	92
Resveratrol	Human aortic smooth muscle	Yes	63
	PC12 rat pheochromocytoma		14
Piceatannol	Bovine aortic endothelial cells	Probable	136
Paclitaxel	Rat vascular smooth muscle cells	Uncertain	18
Rapamycin	Human arterial endothelial and smooth muscle cells	Uncertain	126
Ethyl ferulate	Rat hippocampal neurons and astrocytes	Probable	109
1,2,3,4,6-Penta-O-galloyl-beta-D-glucose	Neuro 2A mouse neuroblastoma	Yes	19
Insulin	ACHN Human renal adenocarcinoma	Yes	46
Isoproterenol	RAW 264.7 Mouse macrophage cell line	Uncertain	116

*Includes simvastatin and lovastatin.

CAPE, Caffeic acid phenethyl ester.

and lovastatin are examples of such compounds. Often, the bio-transformation that occurs *in vivo* is not matched in the experimental system in which the drug is commonly added to a single cell type *in vitro*. This can result in false-negative results. Conversely, false-positive results are also possible, in that compounds that induce HO-1 *in vitro* in cells are ineffective *in vivo*. As an example of the resulting complexity, probucol bisphenol is the major metabolite of probucol *in vivo*, and it induces HO-1 expression in cultured vascular smooth muscles (Fig. 8). In sharp contrast, probucol bisphenol fails to induce HO-1 in vascular smooth muscle cells *in vivo*, whereas both probucol and probucol dithiobisphenol are active (133). This apparent discrepancy between *in vivo* and *in vitro* efficacy presently represents a serious shortcoming in the design of new drugs that may be protective *via* induction of HO-1.

Finally, many drugs are lipophilic, and this commonly represents an obstacle for *in vitro* experiments. The usual strategy to overcome this problem is to add the compound of interest together with a "carrier," such as a protein. However, and as is

often unappreciated, the molar ratio of carrier protein to test compound can importantly affect the outcome. This may be exemplified with probucol. This highly lipophilic compound is commonly added to cells as an albumin complex. However, as can be seen in Fig. 9, the efficacy with which a constant concentration of probucol induces HO-1 in vascular smooth muscle cells varies significantly depending on the molar ratio of albumin to probucol.

The notion of targeting HO-1 to achieve pharmacologic and therapeutic benefit is becoming widely accepted (4, 113). As research continues to uncover positive actions of HO-1 on the maintenance of normal physiology and repair of pathology, increasing numbers of potential applications are likely to be discovered. Simultaneously, the effort to better understand and characterize the molecular mechanism underlying the protective properties of HO-1 will guide the development of novel compounds that harness the body's natural defense systems to combat the diseases they are designed to treat or cure.

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ABBREVIATIONS

AP-1, activator protein-1; CAPE, caffeic acid phenethyl ester; cGMP, cyclic guanosine monophosphate; HO-1, heme oxygenase-1; Keap1, Kelch-like ECH-associated protein-1; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor-kappa B; NO, nitric oxide; NOS, nitric oxide synthase; Nrf2, nuclear factor E2-related factor-2; sGC, soluble guanylate cyclase.

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